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Growth adaptation of probiotics in biopolymer-based coacervate structures to enhance cell viability

L.A. Bosnea [a], T. Moschakis [a], P.S. Nigam [b], C.G. Biliaderis [a, *]

[a] Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki, GR-541 24 Thessaloniki, Greece
[b] School of Biomedical Sciences, University of Ulster, Cromore Rd., Coleraine BT62 1SA, Northern Ireland, United Kingdom

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ABSTRACT

The effect of inoculum size and growth of probiotic cells inside biopolymer-based coacervate structures, made from whey protein isolate and gum arabic, and a dual encapsulation method of complex coacervation coupled with ionotropic gelation on the survival of probiotics was evaluated under adverse environmental conditions; i.e. low pH, heating, and simulated gastric juice (SGJ). The encapsulated bacteria metabolized nutrients and multiplied within the coacervate structural assemblies, pointing to a rather open carrier-delivery system for microbial cells, allowing the exchange of metabolites and nutrients with the bulk liquid medium. Encapsulation of probiotic cells at low counts and subsequent growth improved cell viability upon heating or exposure to SGJ. When cells entrapped in complex coacervates were subsequently embedded in Ca^{2+}-alginate gel microspheres, the remaining viable counts at pH 2.0 for 3 h were even higher by almost 1 logCFU/g. Overall, an initial low inoculum size of bacteria in complex coacervates, followed by culture growth (adaptation stage) and subsequent entrapment in alginate microspheres greatly enhanced the cell viability of probiotic cultures.

1. Introduction

During the past decades, consumer's interest for healthier foods has largely contributed to the development of functional foods that potentially provide additional health benefits apart from the nutrients they deliver (Shiby & Mishra, 2013). Probiotics in particular are considered among the most popular bioactives in formulating functional products, despite the reluctancy of regulatory authorities to approve specific health claims for such microbial cultures. FAO/WHO (2002) defined probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host. The definition itself implies that probiotics need to be alive and present in sufficiently high numbers at the time of consumption to ensure health-promoting effects; i.e. a probiotic product should contain at least 10^6 CFU/g of viable probiotic cells throughout its entire shelf-life (Vasiljevic & Shah, 2008).

Inclusion of probiotics in complex food matrices encompasses several technological challenges (Shah, 2000). A variety of stress factors (e.g. osmotic pressure, heat, low pH, gastrointestinal conditions, reduced water activity, nutrient depletion) during processing and storage as well as upon their transit through the gastrointestinal tract can influence the viability of probiotic bacteria, thus limiting their functional properties. Over the last decade there is ongoing research for development of novel encapsulation methods for microbial cells, including probiotics, to protect them against environmental stresses.

Alginate-based or other types of hydrocolloid matrices are effective carriers of probiotics and prebiotics because of their nontoxicity, biocompatibility/biodegradability, and low cost. Alginates have the ability of forming hydrogels via a mild ionotropic effect (ability of polyelectrolytes to cross link in the presence of counter ions to form hydrogel beads, known as gelispheres) mediated by some divalent cations such as Ca^{2+}. Even though alginates are frequently used for encapsulation of probiotics, conflicting reports exist regarding the protection of bacterial cells against exposure to adverse conditions. Complex coacervation, in particular, has been recently explored as an alternative and quite promising encapsulation method of probiotics (Bosnea, Moschakis, & Biliaderis, 2014). Complex coacervation involving biopolymers consists of spontaneous phase separation by forming an insoluble complex between two or more polymers as a result of mainly electrostatic interactions (Schmitt C., Sanchez, Desobry-Banon, & Hardy, 1998). However, several parameters affect the efficiency of

* Corresponding author.
E-mail address: biliader@agro.auth.gr (C.G. Biliaderis).

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microcapsules production and the variability in their structure such as composition and concentration of biopolymers and environmental conditions (e.g. pH and ionic strength) (McMullen, Newton, & Becker, 1982; Moschakis, Murray, & Biliaderis, 2010; C. Schmitt et al., 2001; Weinbreck, de Vries, Schrooyen, & de Kruif, 2003; Weinbreck, Tromp, & de Kruif, 2004). Microcapsules produced by coacervation are quite adaptable and dynamic metastable structures capable of responding to environmental changes, while they possess excellent controlled-release characteristics (release rate retardation) modulated by changes in ionic strength, pH and temperature (Moschakis et al., 2010).

In a previous study, complex coacervates of whey protein isolate (WPI) and gum arabic (GA) were employed for encapsulation of Lactobacilli cells (Bosnea et al., 2014). This microencapsulation method provided protection to the bacterial cells when exposed to stressful environmental conditions (low pH, gastrointestinal conditions, high salinity and heat treatment). However, other complementary methods need to be explored for further enhancing the viability of the microencapsulated cells in such biopolymer-based coacervate structures.

The aim of the present study was to improve the viability of lactobacillus cells entrapped in complex coacervates of WPI/GA upon exposure to different stress environments. First, the ability of lactobacillus cells to grow and metabolize nutrients within the coacervate structure and then the effect of initial inoculum size on encapsulation yield and on the viability of the encapsulated cells at low pH were investigated. Moreover, the effect of post-culture of probiotic cells encapsulated in low counts in the coacervates on their viability under different stressful environments (heat and simulated gastric juice) was examined. Finally, a dual encapsulation method involving complex coacervation followed by ionotropic gelation in alginate microspheres (Ca^{2+} - induced) was assessed for its effectiveness to improve the viability of the entrapped microbial cells at low pH.

2. Materials and methods

2.1. Materials

Powdered whey protein isolate, WPI Bipro™ (92.08% w/w protein, fat 1.08% w/w, 4.08% w/w ash, 1.08% w/w lactose) was a product of Davisco Foods International Inc. (Le Sueur, MN, USA). The WPI was stored in a hermetically sealed container until use. Lactic acid was purchased from Riedel-de Haën (Hannover, Germany). Gum arabic (GA) was purchased from Sigma Chemicals (Gillingham, UK), whereas monosodium phosphate, disodium phosphate, sodium chloride, potassium chloride, hydrochloric acid and sodium hydroxide were obtained from Merck (Darmstadt, Germany).

2.2. Bacterial strains and growth conditions

The microorganisms used in this study, *L. paracasei* subsp. *paracasei* E6 and *L. paraplantarum* B1, have been isolated from a mature Melichloro cheese (Papanikolaou et al., 2012); the strains were kindly provided by the Laboratory of Food Microbiology and Hygiene, Food Science and Technology Department, Aristotle University of Thessaloniki, Greece. Stock cultures were preserved in De Man, Rogosa and Sharpe (MRS) broth with glycerol (70:30) at −80 °C and were sub-cultured in MRS broth (pH 6.2) for activation prior to the experimental use. The cultures were activated by two successive transfers, then inoculated in 250 mL Erlenmeyer flasks, containing 100 mL of MRS broth, and subsequently incubated for 48 h without agitation at 30 °C.

2.3. Preparation of cells for encapsulation

Harvesting of cells was done by centrifugation at 1800*g* for 10 min. The cell pellets were washed twice with 5 mL of ringer's solution and re-suspended in 2 mL of ringer's solution. Fresh cell cultures were prepared from a stock culture for each one of the replicated experiments (n = 3). Cell density at that point corresponded to ~10^9–10^{10} colony forming units (CFU)/mL.

2.4. Preparation of solutions

Aqueous stock solutions of WPI (3% w/w) and GA (3% w/w) were prepared by dispersing their powders into sterile double distilled water under gentle stirring for 1 h at room temperature to ensure proper dispersion/solublization of the polymeric constituents. The WPI and GA aqueous dispersions were stored overnight at 4 °C to allow complete hydration and solubilisation of the two biopolymers. Both solutions were sterilized by filtration (0.2 μm PTFE filters, BGb Analytik AG, Germany) prior to any use.

Simulated gastric juice (SGJ) was prepared by dissolving pepsin in a 0.5% NaCl (w/v) solution at a concentration of 3 g/L and adjusting the pH at 2.0 with a 1.0 M HCl solution. The mixture was subsequently sterilized through a syringe filter (pore size 0.2 μm).

2.5. Microencapsulation by complex coacervation

The WPI/GA complex coacervates were prepared by blending the two previously prepared solutions of each biopolymer at a weight ratio (WPI:GA) 2:1 and total polymer concentration of 3% (w/v). The cell dispersion was also added to the mixed biopolymer solution and stirred gently for 10 min. The pH of the mixture was then adjusted to pH 4.0 by adding slowly a 10% (v/v) lactic acid solution. The formed coacervates were left to stand at room temperature (20 ± 2 °C) for at least 1 h to allow phase separation. Subsequently, the coacervate phase was obtained by decantation of the clear supernatant. The complex coacervates were then washed twice with 50 ml of Phosphate Buffer Saline (PBS) (pH 4.0) and centrifuged at 100 g for 10 min for removal of free cells (Bosnea et al., 2014). The encapsulation yield was calculated as the total viable cells entrapped in the coacervates divided by the initial number of viable cells introduced in the mixed biopolymer (WPI/GA) solution (initial inoculum size) used for microencapsulation, multiplied by 100; i.e. encapsulation yield (%) = (cells entrapped in coacervates/initial inoculum size) X 100.

2.6. Enumeration of free and of encapsulated bacteria

L. paracasei subsp. *paracasei* E6 and *L. paraplantarum* B1 were enumerated by the pour plate technique on MRS agar. After incubation at 30 °C for 48 h in anaerobic conditions, the total viable counts were measured and expressed as the logarithm of CFU per mL (cell suspension) or g (wet coacervate). The encapsulated cells were first released from the microcapsules by homogenizing 2 g of coacervates in 10 mL of 0.1 M phosphate buffer saline pH 7.4 (PBS); at this pH, the biopolymers in the coacervate structures are no longer interacting electrostatically since they both carry negatively charged groups (repelling forces). The viability of lactobacillus cells was determined by counting viable cells before and after each treatment and expressing them in log scale (Bosnea et al., 2014).

2.7. Confocal microscopy

Samples of 5 mL of the WPI/GA complex coacervates were transferred into a small beaker. A 20 μl aliquot of Nile Blue solution (0.01% w/v) was immediately added and the solution was

thoroughly mixed. Approximately 1 mL of the stained coacervates was then placed into a Willco- Glass bottom dish (0.17 mm thickness) (WillCo Wells BV, Amsterdam, Netherlands) and then examined under the confocal microscope. A Leica TCS SP5II confocal laser scanning microscope (CLSM), mounted on a Leica Model DMI 6000B inverted microscope, was operated in the fluorescence mode with a 60 × oil-immersion objective of numerical aperture 1.40. The images were scanned approximately 20–30 μm below the level of the coverslip to minimize hydrodynamic (and other) interactions with the coverslip. Fluorescence from the sample was excited with the 633 nm of a red HeNe laser line. The signal from the samples was collected and eight scans were averaged for the creation of each image.

A typical confocal micrograph of WPI/GA complex coacervates with entrapped *Lactobacillus paracasei* E6 cells at pH 4.0, 24 h after preparation is presented in Fig. 1A; as shown in this Figure, at pH 4.0, the coacervates formed are ~10–20 μm, with the bacterial cells being distributed throughout the hollow matrix of the coacervate structure.

2.8. Growth kinetics of free and microencapsulated Lactobacillus paracasei by complex coacervation of WPI/GA

L. paracasei E6 cells, encapsulated in WPI/GA complex coacervates or used in a free form (~10^6 CFU/mL), were allowed to ferment a modified MRS broth containing 50 g/L glucose, for 96 h at 37 °C in a shaking bath. The number of viable cells along with the pH of the broth was monitored at 0, 6, 24, 48 and 96 h of fermentation.

2.9. Effect of initial inoculum size on cell viability of encapsulated L. paracasei E6 at low pH

Different levels of inocula of *L. paracasei* E6 were used in either free or encapsulated form in order to obtain coacervates and free cells loaded with 3–11 logCFU/g or mL. The produced coacervates were placed on test tubes containing 10 mL of phosphate buffer saline, adjusted to pH 2.0 with 1.0 M HCl solution. The tubes were incubated at 37 °C and samples were collected for each treatment after 0 and 3 h. The survival rate of the free and the encapsulated cells was evaluated by plate count enumeration on agar, as mentioned in section 2.6.

2.10. Post-culture of microencapsulated probiotic cells within the coacervate structure

Firstly, high cell loads of *L. paracasei* and *L. paraplantarum* were introduced in complex coacervates as described in section 2.5; the initial high cell load was around 8–8.5 logCFU/g of wet coacervate. Secondly, lower cell loads of *L. paracasei* and *L. paraplantarum* in coacervates were also prepared, yielding a ~6 logCFU of encapsulated cells per g of coacervate. For the latter preparations, the entrapped cells in the complex coacervates were further grown for 24 h in an incubator at 30 °C, with the final cell load reaching about 8 logCFU/g after 24 h of incubation. The complex coacervates from both batches were subsequently washed twice with ringer solution (pH 4.0) for removal of fermentation broth and fermentation by-products. Both batches of complex coacervates (with high and low initial load of cells) along with free cells cultures (~8 logCFU/mL) were employed for further treatment, e.g. heat treatment and exposure to simulated gastric juice to evaluate cell viability on a comparative basis under different environmental stresses.

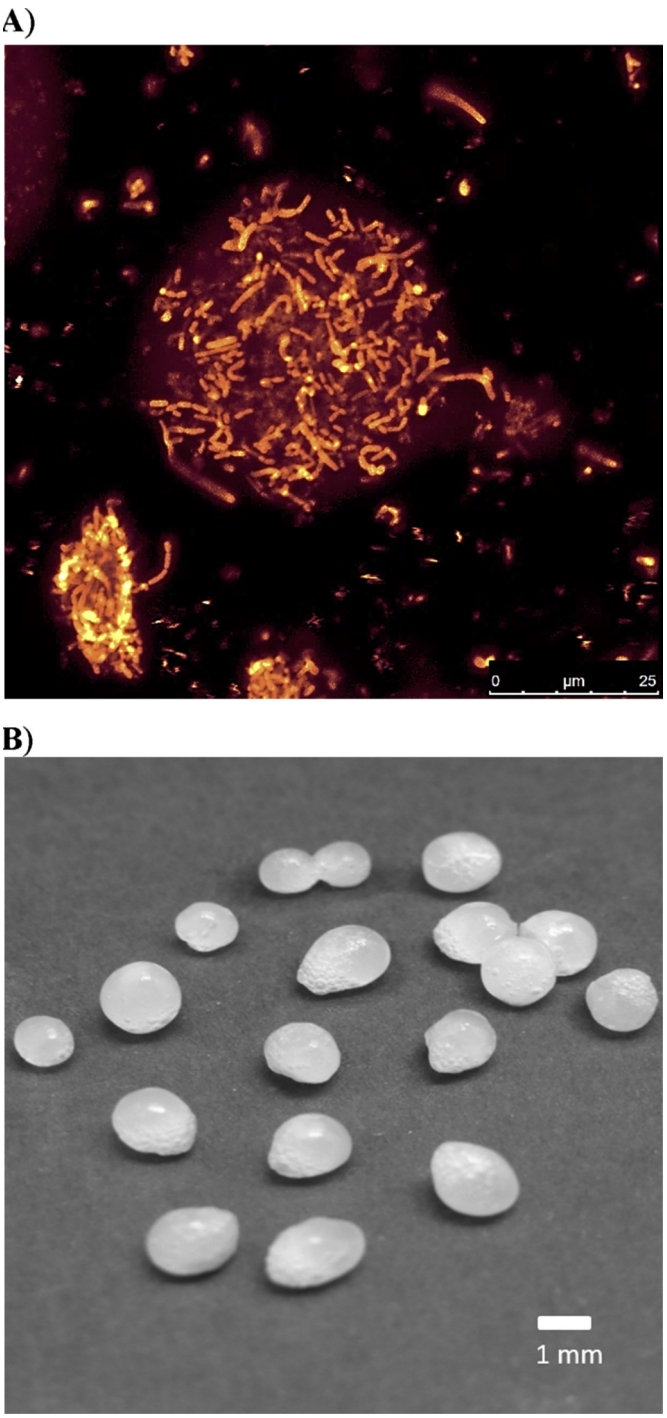

Fig. 1. a) Confocal micrograph of *Lactobacillus paracasei* E6 cells encapsulated in WPI/GA coacervates at pH 4, 24 h after preparation; b) WPI/GA coacervates embedded in alginate gel microspheres.

2.11. Survival of probiotics cultivated in complex coacervates upon heating

The reduction in viable cells of the free and encapsulated (high initial load and post cultured) cells of *L. paracasei* and *L. paraplantarum* upon heating at 65 °C was evaluated according to Bosnea et al. (2014). Specifically, *L. paracasei* subsp. *paracasei* E6 and *L. paraplantarum* B1 free and encapsulated cells were placed into test tubes containing 10 mL of phosphate buffer (0.02 M, pH 7.4,

containing 8.76 g/L NaCl), pH 4.0, at 65 °C. The tubes were sampled after 0, 10, 20 and 30 min and the samples were instantly cooled in an iced water bath before enumeration of the viable cells, as described in section 2.6.

2.12. Effect of exposure of post cultured encapsulated and free bacteria to simulated gastric juice (SGJ)

The reduction in cell viability of free and encapsulated (high initial load and post cultured) *L. paracasei* and *L. paraplantarum* cells during treatment with simulated gastric juice (SGJ) was evaluated according to Bosnea et al. (2014). Briefly, treatment with SGJ was carried out by firstly mixing 2 g of the wet coacervates or 2 mL of washed cell suspension with 10 mL of SGJ (pH 2.0) and immediately placing these suspensions in a shaker incubator at 37 °C for 3 h. Samples were taken at 0 and 3 h for enumeration of live cells according to the method described in section 2.6.

2.13. Encapsulation of complex coacervates with entrapped cells in alginate beads

L. paracasei subsp. *paracasei* E6 cells that were initially encapsulated in complex coacervates were subsequently introduced in alginate gel microspheres. That is, ~2 g of produced complex coacervates were mixed with 8 ml of an aqueous solution of sodium alginate (1% w/v) and stirred at 200 rpm for 5 min. Then, ionotropic gelation was effected by adding dropwise the coacervate–alginate liquid suspension with a syringe (21G, 0.80 × 40 mm) into a $CaCl_2$ (50 mM) solution under stirring. The beads thus formed were stirred for 60min for further hardening, rinsed with sterile double distilled water and then collected by filtration. Fig. 1B illustrates the visual appearance of typical complex coacervates embedded in alginate gel microspheres.

All experiments were carried out in a glass vessel at room temperature. The produced beads were introduced in test tubes containing 10 mL of phosphate buffer saline, adjusted to pH 2.0 with 1.0 M HCl solution. The tubes were incubated at 37 °C and samples were collected for each treatment after 0 and 3 h. Afterwards, an appropriate amount of beads was separated by filtration, washed with sterile double distilled water, and then placed in 100 mL of phosphate buffer, pH 7.0 under stirring for 30min. For enumeration, the partly decomposed beads were further disintegrated by homogenization using an ultra-turrax apparatus at 11000 rpm for 30sec to entirely release the encapsulated cells.

2.14. Statistical analysis

All experiments were conducted at least in triplicate. Statistical analysis was undertaken using one-way analysis of variance ANOVA. The data are presented as mean ± standard deviation (SD) values. Mean multiple comparisons were achieved using Duncan's multiple range test.

3. Results and discussion

3.1. Effect of initial inoculum size on cells viability at pH 2.0

In order to survive and reach the colon in adequate quantities to facilitate their colonization, a large number of probiotic cells must be entrapped in properly designed delivery matrices; i.e. the initial load should be optimized. Increased cell populations could interfere with the capsule materials or in case of complex coacervates could intervene in the complex coacervation process that is mediated via electrostatic interactions. Therefore, different inoculums from 3 up to 10.5 logCFU/g were used for encapsulation of *L. paracasei* E6 cells by complex coacervation in order to determine the effect of the initial inoculum size on cell survival at pH 2.0. Fig. 2 shows the viability of encapsulated *L. paracasei* E6 cell after exposure at pH 2.0 for 3 h as a function of the initial inoculum size. As can be seen in Fig. 2, a higher encapsulation yield was noted at relatively low initial inoculum levels, with a maximum obtained at ~6.5–7.5 logCFU/g initial inoculum size.

As the number of cells encapsulated in the complex coacervates decreased, the reduction of the viable *L. paracasei* cells exposed to low pH conditions also decreased (inset of Fig. 2). This implies that as the relative amount of the biopolymers carrying the cells increased (less number of cells), the effective protection was enhanced. It is noteworthy to mention that when the probiotic population embedded in the coacervate structure was very small (~3 logCFU/g) the cell survival at pH 2.0, upon exposure for 3 h, was ~92.5% (log scale). However, taking into consideration that high numbers of probiotic cells should survive at low pH (e.g. gastric conditions) to exert their beneficial effects, the highest possible initial inoculum size should be used for encapsulation in order to achieve high viable population at low pH, despite the comparative higher % reduction caused by the acid treatment.

3.2. Growth kinetics of free and microencapsulated cells by complex coacervation of WPI/GA in modified MRS broth

In order to study the physiological state of the encapsulated bacteria it was essential to monitor the ability of the bacteria cells to grow within the coacervate structures. For that purpose, 6 logCFU/g of cells (load) were encapsulated in WPI/GA complex coacervates or were used in free form and allowed to ferment a modified MRS broth for 96 h at 37 °C. The number of the viable cells along with the pH change of the broth (acidification) was monitored at 0, 6, 24, 48 and 96 h of fermentation for both cultures (Fig. 3).

It was noted that both free and encapsulated bacteria fermented the broth (glucose) and multiplied. The population of the non-encapsulated bacteria increased almost 1.81 logCFU/mL in 24 h and approximately 3.46 logCFU/mL after 48 h of fermentation, respectively. Interestingly, the encapsulated bacteria grew slightly faster and increased by ~2.66 logCFU/g after 24 h and 4.67 logCFU/g after 48 h of fermentation, respectively. Moreover, a credible difference in ΔpH between the free and the encapsulated cells was recorded after 24 h and this phenomenon became more pronounced as the fermentation time increased. The activity of the free cells seemed to slow down substantially and eventually stopped at pH values around 3.0. On the contrary, the encapsulated cells continued to metabolize the available nutrients, possibly because the coacervate structures provide protection of the entrapped cells against the low pH conditions (Bosnea et al., 2014).

The results of these experiments imply that the coacervate macromolecular assemblies constitute an open system which allows the nutrients to be delivered inside the encapsulated cells and facilitates their growth. Nutrient diffusion within the coacervate is expected to be influenced by the pore size, microheterogeneity of the coacervate lattice, and compactness of its internal structure which can be modulated by pH and ionic strength (Bosnea et al., 2014).

3.3. Survival of cells cultivated in complex coacervates upon heating

One of the important prerequisites for probiotics is that they must survive food production processes. Therefore, the impact of cell growth inside the biopolymer coacervate structures (post-culture) on their survival upon heat treatment at 65 °C for 30 min

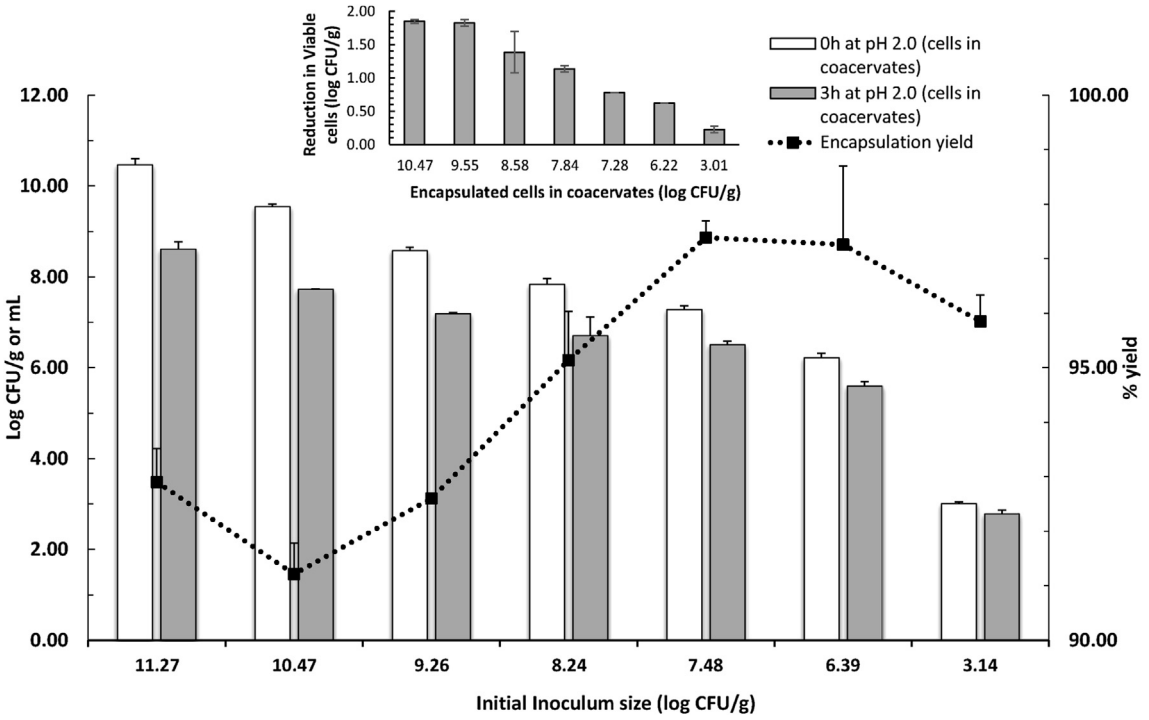

Fig. 2. Cell viability of encapsulated *Lactobacillus paracasei* E6 cells after exposure at pH 2.0 for 3 h as a function of initial inoculum size.

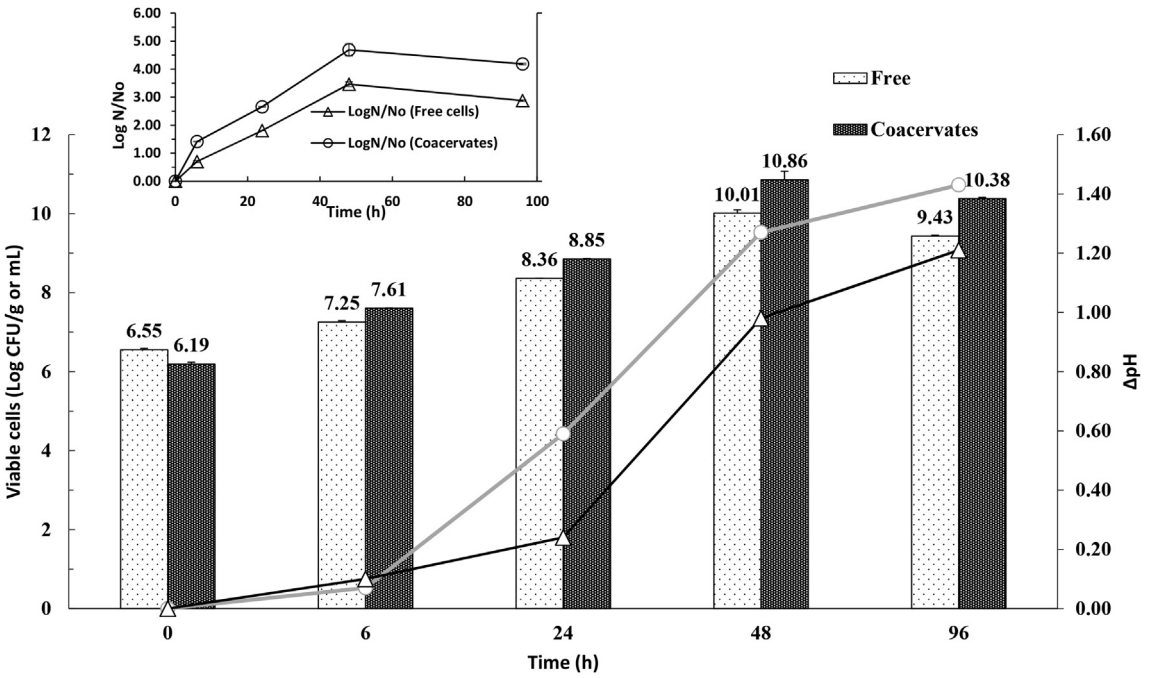

Fig. 3. Growth kinetics of free and microencapsulated by complex coacervation of WPI/GA *Lactobacillus paracasei* E6 cells in modified MRS broth for 96 h; bars represent cell counts and lines the changes in medium pH (ΔpH); ○ cells in coacervate assemblies, **Δ** free cells.

was assessed. More specifically, two different treatments of encapsulation were compared; first, a direct encapsulation of high cell load, and second, the probiotic cells were initially encapsulated in relatively low load numbers and then allowed to reach the same population with the first treatment by growing inside the complex coacervates. The aim was to improve the viability of the cells in harsh environments by allowing them to grow and adapt within the coacervate microenvironment. Fig. 4 shows the effect of growing the cell culture in complex coacervates (GIC) on survival of the *L. paracasei* E6 and *L. paraplantarum* B1 cells upon heat treatment. As can be seen, there was a ~7 logCFU/ml reduction for free cells, ~4 logCFU/g for encapsulated cells of high initial load (C) and ~1 logCFU/g reduction for the encapsulated cells that were post-cultured within the capsules (GIC) for L. *paraplantarum* B1 and

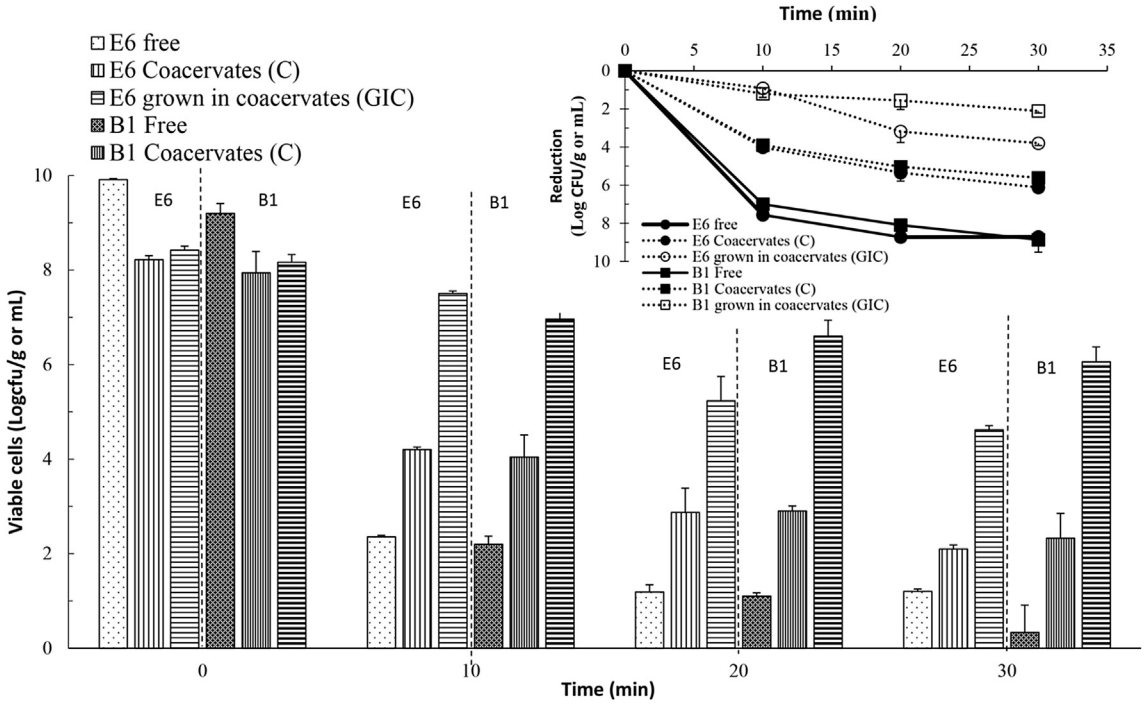

Fig. 4. Effect of post-culture of microencapsulated *L. paracasei* E6 and *L. paraplantarum* B1 cells in coacervate structures on viability during heat treatment at 65 °C for 10–30min.

L. paracasei E6 cells, respectively, after 10min of thermal treatment. It is evident that allowing cell culture growth to occur within the complex coacervate matrix improved the viability by more than 2.7 log CFU/g when a 10 min heating at 65 °C was applied. After 30 min of heat treatment almost all the probiotic cells of the free cells were extinguished, while the cultivation of cells in the coacervate structures (post-culture) positively affected the survival of both strains used. It was of interest also to note that, although the free and the encapsulated cells of both lactobacilli exhibited similar trends in their thermal resistance, the individual strains varied in their survival responses upon heating when they were grown inside the coacervate structures. That is, the B1 showed a smaller cell count reduction than that of E6, indicating that different bacterial strains may possess varying adaptation potential when they move from planktonic type to enclosed conditions mode of culture growth.

3.4. Survival of cells cultivated in complex coacervates at simulated gastric juice for 3 h

Free and encapsulated *L. paracasei* subsp. *paracasei* E6 and *L. paraplantarum* B1 (high and lower initial loads) were also exposed to simulated gastric juice, SGJ (pH 2.0 for 3 h), and the effect of cultivation on survival of probiotic cells was assessed (Fig. 5). After 3 h of treatment in SGJ at 37 °C, there was an 8.7 logCFU/ml reduction for free cells, ~5 logCFU/g for encapsulated cells (C) and 2.5 logCFU/g for encapsulated-adapted cells (GIC) of *L. paraplantarum* and *L. paracasei*, respectively. The cell growth (post-culture) in complex coacervates (GIC) substantially improved the viability of the encapsulated probiotic cells by more than 2 logCFU/g after 3 h treatment with SGJ. These results showed that the harsh gastric conditions significantly affect the survival of all cultures; however, the lactobacillus cells that were allowed to grow in the coacervate structural environment (GIC) were more acid resistant, most likely due to their adaptation.

Song, Yu, Liu, and Ma (2014) also studied the effect of post-culture of yeast cells of low density within alginate-chitosan microcapsules on their survival in different stress environments such as freeze drying, storage and exposure to simulated gastrointestinal fluids. They concluded that probiotic yeast cells which were grown within the alginate–chitosan beads demonstrated higher resistance to stresses, possibly due to better stress adaptation in the microcapsule's environment.

The WPI/GA coacervates are relatively open structures with channels (Bosnea et al., 2014; Moschakis et al., 2010), permitting nutrient delivery, waste-product exchange and some localized cell motility. Additionally, the biopolymers in the composite matrix may also be used from the bacteria as nutrient (carbon) sources. It has been also noted that the complex coacervate structures change dynamically depending on the environmental conditions, e.g. at low pH becoming very dense and more compact (Bosnea et al., 2014). Moreover, when the biopolymers involved in coacervate structures are stiff (e.g. heat-denatured WPI molecules) and the structural rearrangements are lessened, the protective effect of the complex coacervation environment to the entrapped cells is reduced (Bosnea et al., 2014).

It is well known that in assemblies of immobilized bacteria, cell-to-cell interactions are inevitable and the cells become capable of a coordinated and collective behaviour, with different physiological and metabolic responses than their planktonic counterparts, in order to facilitate their adaptation to new environments. In the former case, the cells communicate through signalling molecules and use quorum-sensing to optimize their survival and protection against virulence factors by producing various metabolites (mainly proteins, polysaccharides and lipids) and inducing expression of certain genes and/or other physiological changes in neighbouring cells (Fuqua, Parsek, & Greenberg, 2001; Fuqua, Winans, & Greenberg, 1996; Parsek & Greenberg, 2005). Quorum-sensing is widely recognized as an efficient mechanism responsible for communal behaviours, organization and differentiation in bacteria cells in order to sense-respond to adverse external environment conditions and modulate gene expression accordingly (Daniels,

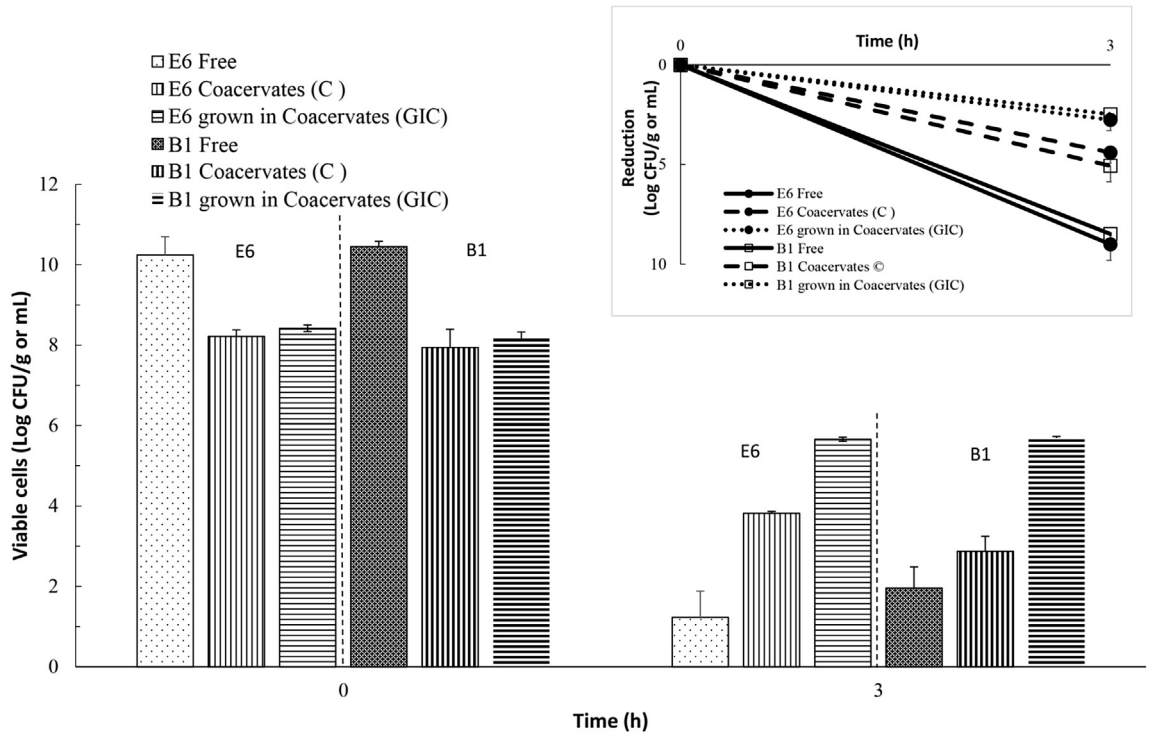

Fig. 5. Effect of post-culture of microencapsulated *L. paracasei* E6 and *L. paraplantarum* B1 cells on viability upon exposure to simulated gastric juice for 3 h.

Vanderleyden, & Michiels, 2004; Fuqua et al., 2001). In our previous work (Bosnea et al., 2014), we also noted that aging of biopolymer coacervates has an influence on their three-dimensional structures via on-going molecular interactions and structural rearrangements of the interacting macromolecules, and this may in turn facilitate the protection and preservation of encapsulated microbial cells, their propagation and probably nutrient delivery to the cellular mass. It seems that apart from the microstructure, the responses of the entrapped cells grown in complex coacervate structures under harsh conditions (low pH, heating) are altered (Figs. 4 and 5); i.e., the biopolymer complex assemblies seem to provide homeostasis in the fluctuating and harsh environmental conditions. For example, at a low pH environment it is possible that binding of hydrogen ions by the polymeric matrix reduces their effective concentration to sublethal levels, thereby offering protection to individual bacterial cells within the coacervate structure compared to free cells.

3.5. *Effect of double encapsulation of complex coacervates in alginate beads on the survival of Lactobacillus paracasei at low pH*

In order to further enhance the viability of *L. paracasei* at low pH, the encapsulated cells by complex coacervation were subsequently embedded in alginate gelispheres and then exposed at pH 2.0 for 3 h to evaluate their viability. As can be seen (Fig. 6), the alginate gel microspheres improved the cell viability by almost 1 logCFU/g, since only a 1.24 logCFU/g reduction was observed at pH 2.0 for 3 h.

The effectiveness of alginate beads in the protection of probiotic bacteria has been extensively investigated in the literature. Previous studies showed that an increment in alginate concentration could provide better protection against environmental factors (Kamalian, Mirhosseini, Mustafa, & Manap, 2014; Trabelsi et al., 2014), mainly by reduced porosity of the bead's matrix. However, the use of alginate is limited due to its low stability in the presence of chelating agents and in acidic conditions below pH 2.0. In the present work, the alginate microspheres also started to deteriorate at low pH values; nevertheless, the beads reduced the negative impact of acids to cell viability. Moreover, it must be noted that the usage of gel microspheres as carriers of probiotic cells may adversely impact the sensorial attributes of the food product. The gel beads produced in the current work were around 0.2–0.3 cm (Fig. 1b). However, the size of the alginate beads can be controlled by manipulating the production parameters that affect the gel bead size; i.e. alginate and cross-linking electrolyte concentrations, liquid droplet size, temperature, pH, etc., with all these parameters affecting reaction rate (cross-linking), shape and size of the microspheres.

Since encapsulation of complex coacervates in a gel matrix has further improved the performance (viability) of the entrapped cells in low pH (Fig. 6), several other gelling biopolymers (e.g. pectins and other polysaccharides, gelatin and dairy proteins), known to cross-link either via ionotropic gelation mechanisms or physical chain segmental associations (H-bonding), could be tested as carriers. However, the increase in production cost of an additional encapsulation step should be assessed in relation to the improved viabilities of probiotic cell cultures for application of such systems in actual food formulations.

4. Conclusions

Two alternative methods for viability enhancement of lactobacilli cells when exposed to different stress environments were developed. First, the probiotic cells were microencapsulated in complex biopolymer coacervate structures at low cell load and allowed for growth therein. Second, complex coacervates with entrapped cells were embedded in alginate gel microspheres to further improve the viability of the probiotic cultures. Both methods clearly showed that microencapsulation of cells by complex coacervation either alone or combined with ionotropic gelation provides significant protection against different environmental

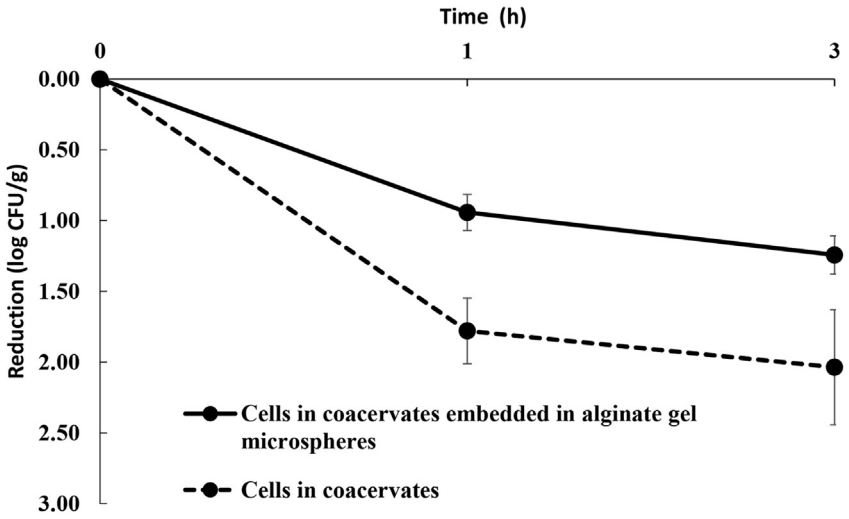

Fig. 6. Effect of coacervate encapsulation in alginate beads (dual encapsulation) on viability reduction of *Lactobacillus paracasei* E6 when exposed to pH 2.0 for 3 h.

factors. Moreover, compared with the planktonic cultures, the lactobacilli cells within the coacervate or the complex coacervate-gel bead structures seem to gain considerable advantages from being part of a community, which provides additional protection against harmful conditions; i.e., communal benefits of multicellular cooperation. These open-structure delivery systems, allowing metabolic activities in the cellular biomass, could be an interesting approach to perform biotransformations involving live microbial cells. From a food product development perspective, it would be necessary to evaluate the cell viability and functionality of these systems in actual food matrices, and test their efficacy under *in vitro* and *in vivo* testing protocols.

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